Altered proliferative responses of dermal fibroblasts to TGF-β_1 may contribute to chronic venous stasis ulcer

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Purpose: **Venous ulcer fibroblasts demonstrate decreased proliferative responses to growth factor stimulation, suggesting cellular senescence. However, the role of chronic venous insufficiency (CVI) disease progression and extracellular matrix (ECM) proteins in agonist-induced cellular proliferation is ill-defined. We hypothesize that CVI-induced fibroblast proliferative resistance to growth factors worsens with disease progression and is regulated by the composition of ECM.**
Methods: **Fibroblast explants were isolated from biopsy specimens from two patients without CVI and 16 patients with CVI of the lower calf (LC) and lower thigh (LT) and stratified according to CEAP disease severity: non-CVI (NC; n = 2), class 2-3 (n = 5), class 4 (n = 5), class 5 (n = 3), and class 6 (n = 3). Proliferation experiments were standardized with a neonatal foreskin fibroblast cell line (HS68). A 10-day course and dose response experiment with 0, 0.5, 1.0, 2.5, 5, 10, and 20 ng/mL of transforming growth factor-β_1 (TGF-β_1) demonstrated maximal cell proliferation at 5 ng/mL of TGF-β_1 on day 4. Under these conditions, CVI dermal fibroblasts were challenged with and without TGF-β_1 and evaluated for proliferative responses on plates coated with polystyrene, collagen, and fibronectin.**
Results: **No differences in unstimulated proliferation were observed in LT and LC fibroblasts from patients with class 2-3 disease and LT fibroblasts from patients with class 4 and 5 disease, compared with NC and HS68 cells. LC fibroblasts from patients with class 4 disease ($P < .05$) and class 5 disease ($P < .001$), and LC ($P < .001$), and LT fibroblasts from patients with class 6 disease ($P < .001$) proliferated to a lesser degree than did NC and HS68 cells. The diminished proliferation observed in class 4 LC cells was reversible with TGF-β_1 stimulation ($P < .004$); however, class 5 and class 6 LC and LT fibroblasts did not respond to stimulation with TGF-β_1. Collagen increased proliferation of HS68 cells with ($P < .05$) and without ($P < .01$) TGF-β_1, compared with cells grown on polystyrene, but did not increase proliferative responses in NC or CVI fibroblasts with and without TGF-β_1. Similarly, fibronectin increased proliferation of HS68 cells ($P < .05$) compared with cells grown on polystyrene, but did not alter proliferation in CVI fibroblasts. Fibronectin did seem to inhibit TGF-β_1-induced proliferation observed in class 4 LC cells.**
Conclusion: **These data indicate that clinical disease progression correlates with cellular dysfunction. Fibroblasts from patients with class 2-3 disease retain their unstimulated and agonist- induced proliferative capacity, compared with NC and HS68 cells. The onset of inflammatory skin changes (class 4 and class 5 disease) diminishes agonist-induced proliferation, and ulcer formation (class 6 disease) severely inhibits it. In addition, the composition of ECM does not affect TGF-β_1-induced proliferation of fibroblasts in CVI. (J Vasc Surg 2003;37:1285-93.)**

Lack of animal models that mimic the dermal sequelae of persistent chronic venous hypertension has resulted in a paucity of information regarding the signaling events that regulate chronic venous insufficiency (CVI)-associated dermal skin conditions. Venous hypertension results in extravasation of red blood cells and macromolecules such as fibrinogen and α_2-macroglobulin into the dermal interstitium of patients with CVI.[1] These cells and molecules are a chronic injury stimulus, resulting in inflammatory leukocyte recruitment and microcirculatory activation. When leukocytes enter the dermal interstitium, transforming growth factor-β_1 (TGF-β_1) and matrix metalloproteinases (MMPs) are released in response to these inflammatory mediators.[2,3] Increased TGF-β_1 activity is associated with CVI disease progression and dermal skin conditions, and fibroblasts are the target cells of leukocyte- derived TGFβ_1.[2,4]

Fibroblasts are activated and proliferate when cultured on polystyrene plates containing growth media with serum. Fibroblast proliferative responses are important in wound healing and fibrogenic pathologic conditions such as systemic sclerosis.[5] Although several cytokines have been implicated in disease states associated with fibroblast proliferation, eg, interleukin-1 (IL-1), tumor necrosis factor-α, platelet-derived growth factor, and fibroblast growth factor, the most potent mediator of cellular proliferation and extracellular matrix (ECM) production is TGF-β_1.[6,7] TGF-βs are a large superfamily of soluble factors important

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in growth, development, and fibroblast proliferation. TGF-β_1 is the isoform usually upregulated in the presence of tissue injury and is the primary mitogenic signal for fibroblasts in wound healing.[5]

ECM is a complex structure of several collagenous and noncollagenous proteins that provides a scaffold for intercellular and intracellular interactions. These interactions are regulated by contact of matrix proteins (integrins) with their corresponding integrin receptors.[8] ECM proteins alone and in conjunction with growth factors can regulate fibroblast proliferation.[8,9] For example, TGF-β_1-induced fibroblast proliferation is augmented by the ED-A domain of fibronectin in a dose-dependent manner.[10] In addition, fibronectin reverses G1 arrest in previously unresponsive fibroblasts.[9] The role of ECM proteins and TGF-β_1 on CVI fibroblast proliferation is ill-defined. We previously demonstrated that CVI disease progression is associated with increased tissue levels of active TGF-β_1. In addition, CVI fibroblasts derived from venous ulcer explants demonstrate diminished proliferation.[11-13] We therefore hypothesized that TGF-β_1-induced fibroblast proliferative responses in CVI are regulated by the composition of ECM and are altered with disease progression.

METHODS

Patient selection and disease severity classification. Sixteen patients with CVI were included in the study. To ensure that the observed measurements were secondary to CVI alone, patients were excluded if they had active infection, cancer, rheumatoid arthritis, vasculitis, or collagen vascular disease; had undergone surgery within 6 weeks; used steroid medications; or had a history of or current use of intravenous drugs. Two patients without CVI were also included in the study as non-CVI (NC) controls. Informed consent was obtained from each patient, and the research protocol was approved by the institutional review boards of UMDNJ-New Jersey Medical School and the East Orange Veterans Affairs Medical Center.

All patients with CVI and control subjects without CVI underwent venous duplex scanning with a 5 MHz imaging/5 MHz pulsed-wave Doppler color-flow system (Quantum 2000; Siemens, Seattle, Wash) to confirm the presence or absence of venous reflux and extent of disease. In addition, the site of valvular incompetence, pattern of reflux, and location of chronic venous obstruction in deep and superficial systems were also determined.[14] Most patients had bilateral lower extremity venous disease, and assignment to a clinical group was based on the more severely affected limb.

Punch biopsy specimens were obtained from the affected lower calf (LC; disease samples) and from the lower thigh (LT) of 16 patients (internal control subjects). One biopsy specimen each was acquired from the lower thigh of 2 subjects without CVI (NC control samples) who were undergoing bypass procedures because of arterial insufficiency. Biopsy specimens were stratified according to The American Association for Vascular Surgery/Society for Vascular Surgery (AAVS/SVS) CEAP disease classification for CVI[15]: NC (n = 2), class 2 or 3 (n = 5), class 4 (n = 5), class 5 (n = 3), or class 6 (n = 3).

Biopsy protocol. Biopsy specimens were obtained as described.[4] In brief, after injection of 1% lidocaine, two 6.0 mm punch biopsy samples were obtained from areas that demonstrated clinical signs of venous stasis dermatitis in the LC and two from clinically normal skin in the ipsilateral LT. Biopsy sites were closed with single 2-0 prolene sutures, and dressings were applied. Sutures were removed at 1-week follow-up assessment of the wound. In patients undergoing vein stripping or perforator ligation, biopsy tissue was obtained from incision sites made during surgery. Thigh biopsy specimens from clinically normal skin, obtained from patients undergoing infrainguinal revascularization surgery, served as NC controls. Calf biopsy samples were not obtained from this group because of the possibility of inducing wound complications.

Primary fibroblast culture protocol. Dermal fibroblasts were isolated from explanted biopsy specimens. Skin biopsy tissue was processed immediately after harvesting. Subcutaneous fat and epidermis were excised, and explants were cut into approximately 1 mm^3 pieces. Tissue explants were disinfected with three sequential washes with full-strength povidone iodine (5 minutes), 70% ethanol (5 minutes), and 250 μg/mL of amphotericin-B (10 minutes). The interstitial and remaining epidermal elements were digested with 0.25% trypsin at 4°C 18 hours, followed by 30 minutes of digestion at 37°C. The explants were then seeded onto 60 mm etched culture plates and cultured with Dulbecco's modified Eagle's media (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1 μg/mL of amphotericin-B (all culture media from Gibco BRL, Rockville, Md). The media were first changed at week 1 and every 2 days thereafter. Most explants demonstrated growth of fibroblasts within 2 weeks (Fig 1), at which point explants were removed. Cells were trypsinized at 50% to 70% confluence. Half of the cells were frozen in dimethyl sulfoxide solution in liquid nitrogen for future use. The remaining cells were replated and used for proliferation experiments. All experiments were performed on passage 3 or less cells.

Time course and dose response of fibroblasts to TGF-β_1. The optimal dose and time for TGF-β_1-induced proliferative responses of fibroblasts were standardized with a commercially available neonatal foreskin fibroblast cell line (HS68; American Type Culture Collection, Rockville, Md). HS68 cells from passages 2 to 5 were seeded into 24-well polystyrene plates at a density of 15,000 cells per well. The cells were serum starved for 18 hours with DMEM alone, then cultured in DMEM containing 10% FBS with or without TGF-β_1 (Sigma, St Louis, Mo). The cells were then trypsinized and stained with trypan blue to determine viability, and were manually counted with a hemocytometer. These measurements were performed daily for 10 days. Linear regression analysis was performed to determine logarithmic cell growth period for fibroblasts stimulated with TGF-β_1. The experiment was repeated in a representative sample of fibroblasts derived from a patient

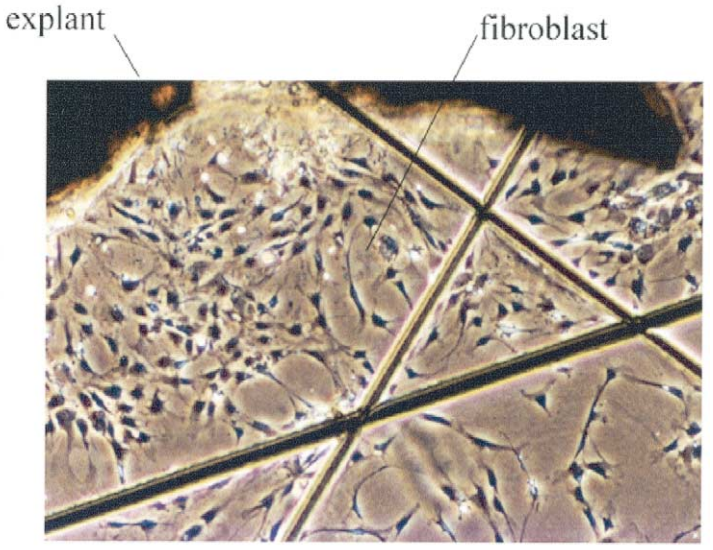

Fig 1. Dermal fibroblasts were cultured from lower extremity skin biopsy specimens (see Methods). In 2 to 3 weeks, patient fibroblasts could be seen migrating from the explant and populating the culture dish.

with CVI to confirm that adult fibroblasts followed the same proliferation kinetics as the neonatal cells did.

Once the logarithmic period had been determined, HS68 cells were exposed to increasing doses of TGF-β_1 (0.5, 2.5, 5, 10, and 20 ng/mL) in a separate experiment. Cell count and viability assessments were performed for each dose during the logarithmic period of growth to determine the dose responsible for maximal TGF-β_1–induced proliferation.

Proliferative response of patient fibroblasts. Primary cultures from 16 patients with CVI patients, 2 patients without CVI, and HS68 cells were then subjected to the following experiments. Fibroblasts were seeded into 24-well plates at a density of 15,000 cells per well. Cells were cultured with and without TGF-β_1 and counted during the logarithmic period, as determined in the previous experiments. All experiments were first performed with patient fibroblasts growing on polystyrene 24-well plates without TGF-β_1 to determine baseline unstimulated proliferation. The proliferative response to TGF-β_1 was subsequently evaluated. Patient fibroblasts were then seeded onto 24-well plates coated with type I collagen (BioCoat; Becton Dickinson, Franklin Lakes, NJ) and fibronectin (BioCoat) with and without TGF-β_1, and proliferative responses were measured.

Statistical analysis. A minimum of four wells per explant were tested under each condition. Differences in mean ± SEM between groups were analyzed with one-way analysis of variance and the Bonferroni post hoc test. Differences within groups were analyzed with a two-tailed unpaired *t* test. Significance was accepted at $P \leq .05$. All statistical analyses were performed with GraphPad Prism (version 3.00; GraphPad Software, San Diego, Calif).

Table I. Associated medical comorbid conditions

	Patients without CVI (n = 2)	*Patients with CVI (n = 16)*
Diabetes	0	0
Stroke	0	1
Coronary artery disease	1	1
Hypertension	1	3
Tobacco use	2	7
COPD	0	0
Deep vein thrombosis	0	0

CVI, Chronic venous insufficiency; *COPD,* chronic obstructive pulmonary disease.

RESULTS

Patient demographics. Thirteen men and 5 women were enrolled in the study. Their mean age was as follows: NC control subjects, 54 years (range, 50-58 years); patients with class 2-3 disease, 35.2 years (range, 20-54 years); patients with class 4 disease, 57.4 years (range, 37-71 years); patients with class 5 disease, 58.6 years (range, 38-80 years); and patients with class 6 disease, 53.9 years (range, 50-82 years). Associated medical comorbid conditions are presented in Table I. The clinical, etiologic, anatomic, and pathophysiologic distribution of CVI was determined clinically and with duplex ultrasonographic (US) scanning as per the recommended reporting standards of the AAVS/SVS. The distribution of reflux on duplex US scans is summarized in Table II. Post-phlebitic vein wall changes were not noted in any patients.

Proliferation kinetics. Unstimulated neonatal fibroblasts (HS68) proliferated on polystyrene plates, and the rate was significantly increased when challenged with 5 ng/mL of TGF-β_1 ($P < .05$). Linear regression analysis of

Table II. Description of reflux with duplex scanning

	Class 2-3	*Class 4*	*Class 5*	*Class 6*
Superficial	2	2	0	0
Superficial, deep	0	0	1	1
Superficial, perforator	2	2	2	1
Superficial, deep, perforator	1	0	1	1
Postphlebotic	0	0	0	0

the growth curve demonstrated logarithmic cell growth between days 2 and 7 (Fig 2, *A*). The proliferation kinetics of a primary fibroblast culture from a patient with CVI CEAP class 6 was studied. We chose CEAP 6 patient fibroblasts because they could be expected to have the slowest rate of proliferation and therefore significantly different growth kinetics. This study would enable us to identify the appropriate time in the mid-logarithmic phase to conduct future experiments. The growth curve of the CEAP 6 cells demonstrated a similar logarithmic phase between days 2 and 7, although with a significantly lower slope than that of HS68 cells ($P < .05$; Fig 2, *B*). Proliferation counts were therefore performed on day 4, in mid-logarithmic phase, for all further experiments.

Dose response. HS68 fibroblasts were grown in the presence of increasing concentrations of TGF-β_1, and proliferation was assessed on day 4. Proliferation was maximal at a dose of 5 ng/mL ($P < .05$; Fig 3). TGF-β_1 concentrations of 0.5 and 20 ng/mL did not cause increased proliferation. TGF-β_1 at 5 ng/mL was used for all further proliferation experiments.

Baseline unstimulated proliferation of patient fibroblasts according to disease severity. HS68, NC, and CVI fibroblasts were grown on polystyrene plates, and cell counts were performed on day 4 (Fig 4). Percent cell viability was noted to be greater than 95% in all cases. NC fibroblasts proliferated to the same degree as HS68 cells under these baseline conditions ($P > .05$). Similarly, LT and LC fibroblasts from patients with CEAP 2-3 CVI and LT fibroblasts from patients with CEAP 4 and 5 CVI proliferated as well as did NC and HS68 cells ($P > .05$ in all instances). However, LC fibroblasts from patients with CEAP 4 CVI demonstrated significantly lower proliferation ($P < .0$ 1 vs HS68, and $P < .001$ vs LT cells of same class). Similarly, LC fibroblasts from patients with CEAP 5 CVI proliferated less than did HS68 cells ($P < .05$) and their LT counterparts ($P < .01$). Conversely, both, LT and LC fibroblasts from patients with CEAP 6 CVI proliferated to a lesser degree (vs HS68 cells, $P < .001$ for both; and vs NC cells, $P < .001$ for both).

Proliferation response to TGF-β_1 according to disease severity. HS68, NC, and CVI fibroblasts were grown on polystyrene plates, and their response to 5 ng/mL of TGF-β_1 stimulation was assessed (Fig 5). HS68 cells demonstrated increased proliferation with TGF-β_1 above their unstimulated baseline value ($P < .05$). NC cells demonstrated a trend toward increased proliferation with TGF-β_1 but did not reach statistical significance. Both LT and LC fibroblasts from CEAP 2-3 CVI demonstrated increased proliferation with TGF-β_1 compared with their unstimulated baseline value ($P < .0$ 5 for both). Of note, LC fibroblasts from CEAP 4 CVI demonstrated increased proliferation with TGF-β_1 ($P < .05$) compared with unstimulated CEAP 4 CVI cells. LT and LC fibroblasts from patients with CEAP 5 disease did not demonstrate increased proliferation in response to TGF-β_1 compared with their respective unstimulated baseline value. CEAP 6 LT and LC fibroblasts demonstrated significantly diminished unstimulated proliferation at baseline compared with both HS68 and NC cells, and proliferation did not increase in response to TGF-β_1 stimulation.

Proliferation response to collagen and fibronectin. HS68, NC, and CVI fibroblasts were grown on plates coated with polystyrene, type I collagen, and fibronectin with and without TGF-β_1 stimulation, and cell counts were performed on day 4. Unstimulated HS68 fibroblasts (Fig 6, *A*) demonstrated increased proliferation when grown on collagen ($P < .01$) and fibronectin ($P < .001$) compared with polystyrene. However, proliferation of NC fibroblasts was not increased with either ECM protein. Similarly, proliferation of LC and LT fibroblasts from any CVI class was not increased in the presence of ECM proteins when compared with polystyrene.

TGF-β_1 stimulation (Fig 6, *B*) increased proliferation of HS68, NC, and CEAP 2-3 LT and LC fibroblasts above their respective unstimulated baseline value. However, no synergistic or additive proliferative responses were observed when these experiments were repeated with cells grown on ECM proteins (collagen or fibronectin). CEAP 5 and 6 LT and LC fibroblasts stimulated with TGF-β_1 exhibited no increase in proliferation compared with their respective unstimulated baseline value whether cultured on polystyrene, collagen, or fibronectin. ECM proteins did not seem to alter the response of patient fibroblasts to TGF-β_1 when compared with cells grown on polystyrene. The only exception to this trend was LC fibroblasts from patients with CEAP 4 disease. These cells did not proliferate in response to TGF-β_1 in the presence of fibronectin compared with polystyrene ($P < .05$).

DISCUSSION

CVI disease progression is characterized by an intense leukocyte inflammatory response, ECM deposition, changes in cellular architecture, and tissue fibrosis indicating an imbalance between tissue degradation and repair.[15-18] In CVI, fibroblasts are central to the processes described above.[5,6,17] They are the target cells of leukocyte-derived TGF-β_1, principal producers of ECM proteins, and are intimately involved in wound contraction and healing.[5,11]

Of the known cytokines, TGF-β_1 is the prototypical regulator of fibroblast activation and proliferation.[5-7,19] In patients with CVI the source of TGF-β_1 seems to be dermal leukocytes, with interstitial collagen matrix serving as an important repository of latent TGF-β_1.[4,20] We previously demonstrated a correlation between local dermal TGF-β_1

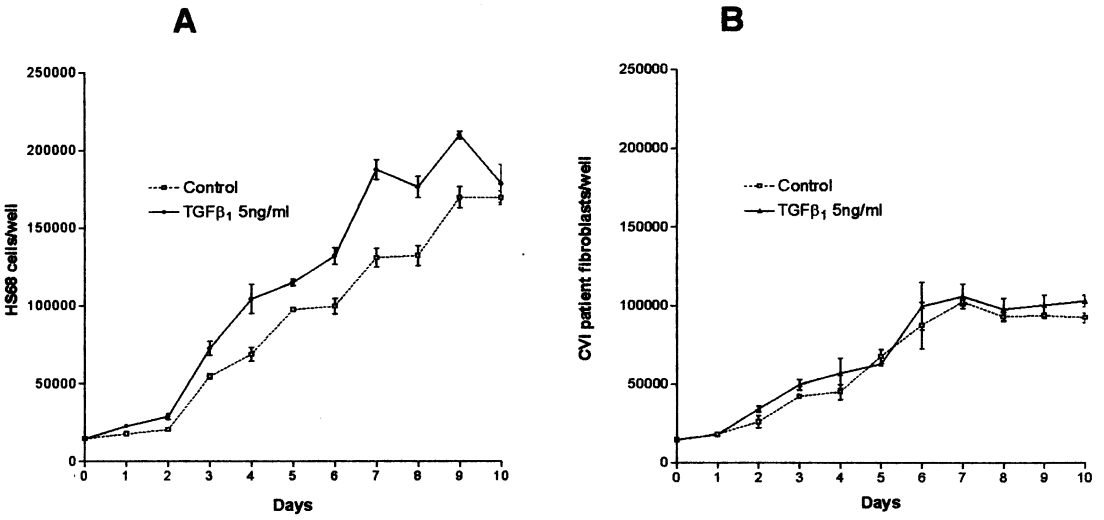

Fig 2. Logarithmic growth phase kinetics. Proliferation response of fibroblasts to 5 ng/mL of TGF-β_1 over 10 days. **A,** Response of HS68 fibroblasts. **B,** Response of fibroblasts from a patient with CEAP class 6 CVI. On linear regression analysis the logarithmic period of growth extended from days 2 to 7 in both cases.

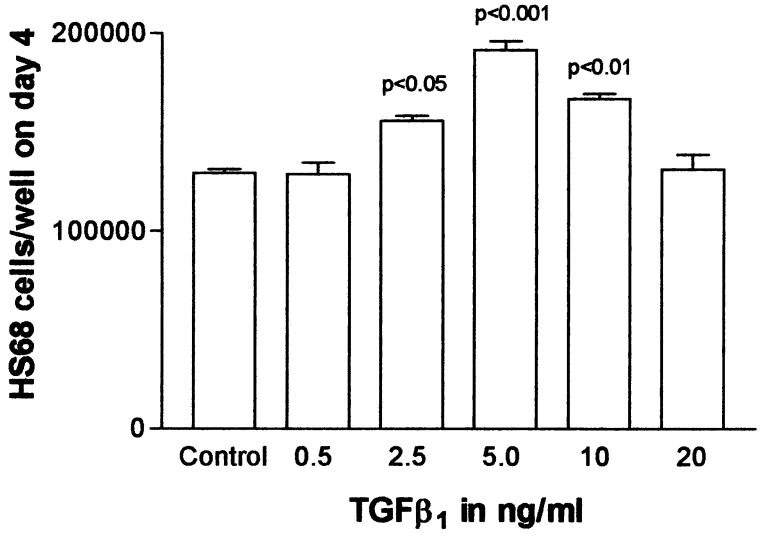

Fig 3. Dose response. Proliferation response of HS68 fibroblasts to increasing concentrations of TGF-β_1. Fibroblasts responded with increased proliferation at TGF-β_1 concentrations of 2.5 ng/mL ($P < .05$), 5 ng/mL ($P < .001$), and 10 ng/mL ($P < .01$).

tissue levels and CVI disease progression.[2] Increasing protein concentrations of active TGF-β_1 were noted in dermal biopsy specimens with increasing CEAP severity of disease (healthy skin, <1.0 ng/g; class 4, 5.01 ng/g; class 5, 8.3 ng/g; and class 6, 5.4 ng/g).[2] The biologic activity of TGF-β_1 is closely controlled by its concentration. Femtomolar concentrations are strongly chemotactic for leukocytes and fibroblasts, whereas low nanomolar concentrations activate monocytes and fibroblasts. In addition, high nanomolar concentrations may inhibit fibroblast activation and proliferation. The mechanism of this concentration effect on cellular proliferation has not been determined. In the present study, we systematically performed a dose-response and time course experiment with TGF-β_1 on fibroblasts and found that the maximal in vitro proliferative response to TGF-β_1 occurs at the same concentrations present in the tissues of patients with advanced CVI. This observation suggests that tissue concentrations of TGF-β_1 observed in severe CVI (CEAP 4-6) could induce significant fibroblast proliferative responses.

The "dual control model of proliferation" suggests that optimal cellular replication requires the presence of a

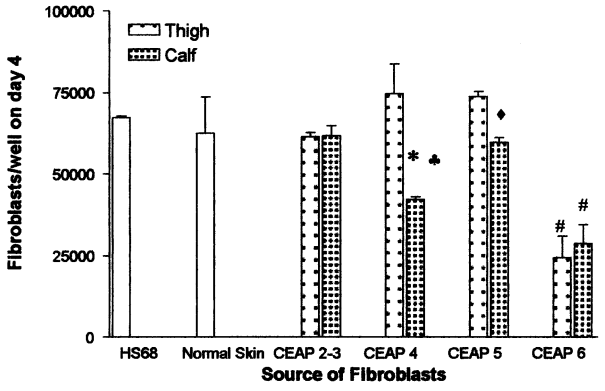

Fig 4. Baseline unstimulated proliferation of fibroblasts derived from HS68 cell line, NC skin, and patients with CVI. LT and LC fibroblasts from patients with CEAP 2-3 disease demonstrated proliferation similar to that of HS68 and NC adult skin fibroblasts. CEAP 4 LC fibroblasts proliferated less compared with LT cells ($^{*}P < .001$) and HS68 cells ($\clubsuit P < .05$). LC CEAP 5 fibroblasts proliferated less than LT cells did ($\blacklozenge P < .001$). LC fibroblasts from patients with CEAP 4 and 5 CVI proliferated to the same degree as HS68 did. However, both LT and LC fibroblasts from CEAP 6 CVI proliferated less than did HS68 and NC adult skin cells ($^{\#}P < .001$ for both).

growth factor as well as a coactivator. Recent investigations have demonstrated that, in the case of fibroblasts, ECM can function as a coactivator.[9,21] In the presence of a fixed dose of TGF-β_1, both collagen and fibronectin stimulate fibroblast replication in a dose-dependent manner.[9,10,21] Signaling between ECM and fibroblasts occurs through integrin receptor stimulation at the surface of the cell (collagen, mainly $\alpha_1\beta_1$ and $\alpha_2\beta_1$; fibronectin, mainly $\alpha_3\beta_1$).[11] Clinically and histologically, intense ECM deposition has been observed in the lower extremities of patients with CVI, suggesting a coactivator role for these proteins in CVI disease progression.[4,22] We anticipated that excessive TGF-β_1 tissue levels, in conjunction with increased ECM proteins, would result in increased CVI fibroblast proliferation.[2,6] However, several reports have noted that fibroblasts cultured from CVI ulcer edge explants demonstrate diminished baseline and agonist- induced proliferative responses.[7] These investigations have suggested that cellular senescence is a possible cause for these observations[12,13]; however, the cytochemical component responsible for these phenotypic alterations in CVI fibroblasts remains unknown. Similarly, the relationship of these proliferation abnormalities to CEAP disease severity is ill-defined.

Fibroblasts cultured from patients with early manifestations of CVI (CEAP class 2-3) and LT fibroblasts from patients with class 4 and 5 CVI proliferated to the same extent as did HS68 and NC fibroblasts under baseline conditions. However, fibroblasts from areas of clinically evident disease (LC) in class 4 and 5 CVI demonstrated reduced baseline proliferation, as did class 6 fibroblasts from both LT and LC explants. We therefore observed progressive worsening of baseline proliferation with disease progression: normal proliferation in class 2-3; regional variation between LT and LC in class 4 and class 5 disease; and proliferative deficiency throughout the entire lower extremity, not limited solely to the ulcer edge, in class 6 disease.

When stimulated with 5 ng/mL of TGF-β_1, neonatal foreskin and class 2-3 fibroblasts demonstrated significantly increased proliferative responses above their respective baseline value. It is clear that fibroblasts in this stage of disease retain their ability to respond to mitogenic stimuli. There was a trend toward proliferation of NC fibroblasts exposed to TGF-β_1 above their unstimulated baseline value, but the result was not statistically significant. This is probably related to the limited number of patients in this group; more subjects in this group could potentially strengthen the conclusions. Of note, TGF-β_1 treatment resulted in the rescue of fibroblasts derived from the LC in patients with class 4 CVI. These cells demonstrated significantly increased proliferation above their baseline value after treatment with TGF-β_1. This was the only group of fibroblasts that demonstrated a rescue response unlike that of class 5 and 6 LT or LC cells. Our results demonstrate that proliferative resistance to TGF-β_1 characterizes class 5 and 6 fibroblasts, of which patients with class 6 CVI are most severely affected. Furthermore, this proliferative resistance may not be limited to regions of clinically active disease, indicating a more global proliferative defect.

Progressive loss of proliferative capacity in patients with venous ulcer may also explain why trials with direct application of TGF-β_1 to CVI-associated ulcers have failed to substantially accelerate healing.[23,24] One group has studied agonist-induced proliferation of fibroblasts cultured from CVI ulcer edges and noted variable results. Proliferation was augmented by basic fibroblast growth factor and epidermal growth factor in most but not all fibroblast cultures. IL-1β had no effect on proliferation. Although these cytokines are mitogenic to fibroblasts, they are not the primary agonists for fibroblast activation and proliferation, and their protein expression has not been reported to be altered in CVI affected limbs. Trials using local application of these growth factors for CVI ulcer healing have resulted in marginal success at best.[25,26]

Although progressive proliferative resistance to TGF-β_1 may explain the low baseline proliferation of class 4 to class 6 CVI, the mechanism for this resistance remains unknown. Since fibronectin and type I collagen are expressed in abundance during different stages of wound healing and granulation tissue formation, we tested the hypothesis that ECM proteins may modulate the proliferative action of TGF-β_1 on CVI fibroblasts. In the absence of TGF-β_1, collagen increased baseline proliferation of neonatal fibroblasts but not of fibroblasts isolated from adults with NC or patients with CVI. TGF-β_1 stimulation in the presence of collagen did not result in additional proliferation of neonatal or patient fibroblasts beyond that seen with TGF-β_1 on polystyrene, indicating that collagen matrices in these experimental conditions did not augment

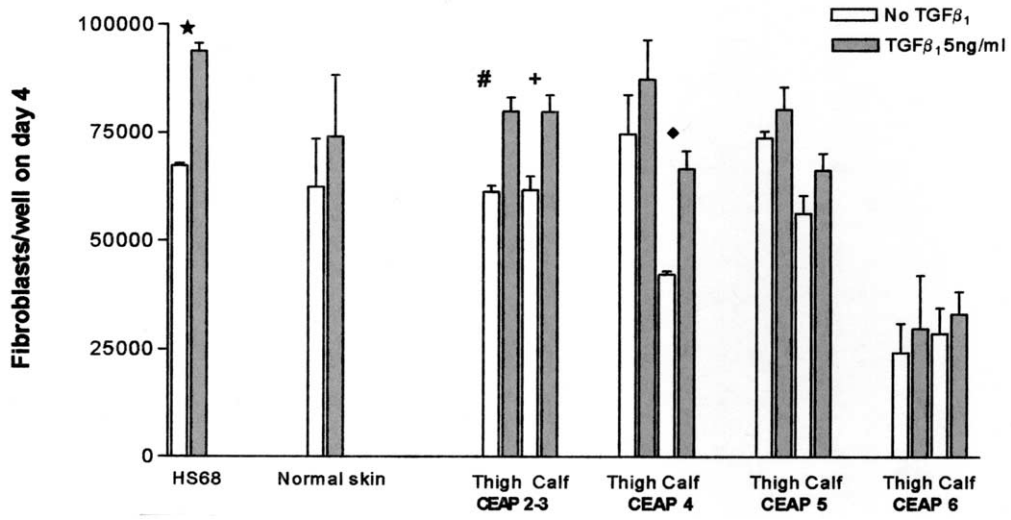

Fig 5. Proliferation response to TGF-β_1 (5 ng/mL) of fibroblasts derived from HS68 cell line, NC skin, and patients with CVI. HS68 cells ($^{*}P < .001$), LT fibroblasts ($^{\#}P < .001$), and LC fibroblasts ($^{+}P = .007$) from patients with CEAP 2-3 CVI and LC fibroblasts (♦P = .004) from patients with CEAP 4 CVI all demonstrated increased proliferation when exposed to TGF-β_1. However, LC and LT fibroblasts from patients with CEAP 5 and 6 CVI did not exhibit increased proliferation in response to TGF-β_1.

the mitogenic action of TGF-β_1 on patient fibroblasts grown as a monolayer. Although most studies addressing fibroblast proliferation have been conducted in vitro with monolayer cultures, in vivo skin fibroblasts are surrounded by dermal ECM proteins in a three-dimensional (3D) arrangement. To study fibroblast behavior in a situation resembling the in vivo environment more closely, experiments have been conducted with 3D collagen matrix gels. Investigations with fibroblasts isolated from hypertrophic scars[27] demonstrated that dermal fibroblasts can reorganize collagen matrix to cause contraction. The subsequent tension within the matrix causes substantial fibroblast proliferation. This mechanism may be involved in the genesis of hypertrophic scars occurring at flexural surfaces of high skin tension.[28] Conversely, the mechanism for an observed increase in proliferation of fibroblasts in scleroderma[29] appears to be upregulation in the number of TGF-β_1 receptors on the cell surface. These cells do not demonstrate increased proliferation within 3D collagen gels compared with monolayers grown on collagen. In the current study, TGF-β_1 in conjunction with collagen did not result in additive or synergistic increases in fibroblast proliferation. Possible causes of this phenomenon are need for a 3D ECM configuration, downregulation of TGF-β_1 type I or II receptors in CVI, and altered integrin receptor signaling in CVI.

Like collagen, fibronectin can serve as a fibroblast growth factor and coactivator of fibroblast proliferation in conjunction with other growth factors.[9] Both intact fibronectin molecules and their fragments demonstrate functional activity.[30] Fibronectin may contribute to transformation of vascular smooth muscle cells from a contractile to a proliferative phenotype in conditions such as atherosclerosis, restenosis, and hypertension.[31,32] The end organ damage seen in the heart in hypertension is characterized by a marked increase in fibronectin expression and smooth muscle cell proliferation.[31,32] Tissue injury can give rise to several splice variants of fibronectin, of which the ED-A isoform promotes fibroblast proliferation in granulation tissue, cirrhosis, pulmonary fibrosis, and other fibrogenic diseases.[32] In this study, fibronectin functioned as a growth factor for neonatal fibroblasts. The proliferation of non-CVI adult cells and patient fibroblasts across all disease classes, however, remained unchanged from their respective baseline value. On stimulation with TGF-β_1, LC fibroblasts from patients with class 4 disease that had previously been rescued by TGF-β_1 failed to exhibit increased proliferation. This result may be of possible significance, but there was no consistent trend among other classes of fibroblasts. The absence of a permissive or promoter role of fibronectin in CVI could be secondary to culturing cells under monolayer conditions. Alternatively, the appropriate fibronectin splice variant that promotes proliferation may not have been present in our monolayer culture system or actively degraded in CVI-affected tissue.

In summary, our data indicate that clinical disease progression correlates with cellular dysfunction. Baseline proliferation of fibroblasts in patients with CVI progressively diminishes with increasing disease severity. ECM does not appear to augment agonist-induced cellular proliferation. The cause of CVI fibroblast proliferative resistance remains unknown. Therapeutic methods to accelerate ulcer healing in patients with CVI will benefit

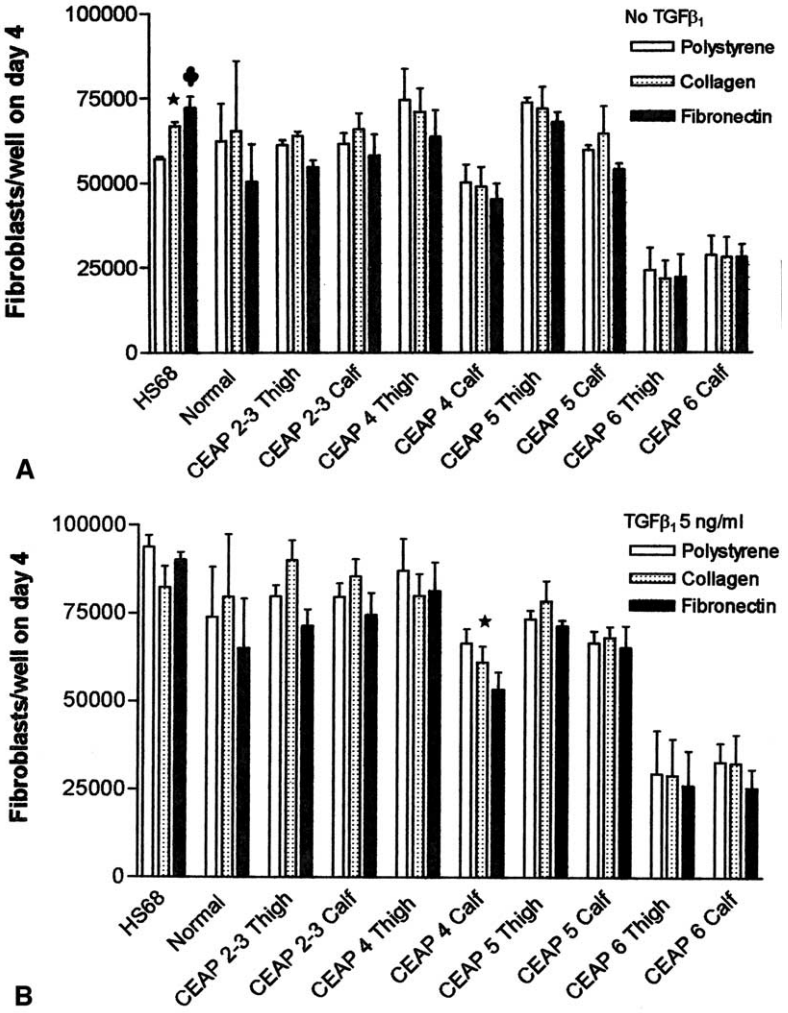

Fig 6. Proliferation response of fibroblasts grown on plates coated with polystyrene, collagen, and fibronectin. **A,** Without TGF-β_1. HS68 fibroblasts demonstrated increased proliferation when grown on collagen (*$P < .01$) and fibronectin (♣$P < .001$) compared witt polystyrene. Proliferation of NC, LC, and LT fibroblasts in any CVI class was not increased by these ECM proteins when compared with growth on polystyrene. **B,** With TGF-β_1 (5 ng/mL). TGF-β_1 increased proliferation of HS68, NC, and CEAP 2-3 CVI fibroblasts when cells were cultured on collagen and fibronectin to the same extent as when these cells were grown on polystyrene. TGF-β_1 did not increase proliferation of CEAP 4-6 LC fibroblasts grown on collagen or fibronectin, but the response was similar to that of cells grown on polystyrene. LC CEAP 4 fibroblast proliferation was inhibited in the presence of fibronectin (*$P < .05$) compared with cells cultured on polystyrene.

substantially from additional information regarding mechanisms that regulate proliferative resistance of fibroblasts.

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